Protein Translocation through Anthrax Toxin Channels Formed in Planar Lipid Bilayers

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ABSTRACT The 63-kDa fragment of the protective antigen (PA) component of anthrax toxin forms a heptameric channel, $(PA_{63})_7$, in acidic endosomal membranes that leads to the translocation of edema factor (EF) and lethal factor (LF) to the cytosol. It also forms a channel in planar phospholipid bilayer membranes. What role does this channel play in the translocation of EF and LF? We report that after the 263-residue N-terminal piece of LF (LF_N) binds to its receptor on the $(PA_{63})_7$ channel and its N-terminal end enters the channel at small positive voltages to block it, LF_N is translocated through the channel to the opposite side at large positive voltages, thereby unblocking it. Thus, all of the translocation machinery is contained in the $(PA_{63})_7$ channel, and translocation does not require any cellular proteins. The kinetics of this translocation are S-shaped, voltage-dependent, and occur on a timescale of seconds. We suggest that the translocation process might be explained simply by electrophoresis of unfolded LF_N through the channel, but the refolding of the N-terminal half of LF_N as it emerges from the channel may also provide energy for moving the rest of the molecule through the channel.

INTRODUCTION

A number of bacterial toxins consist of two components: an enzymatic A moiety, and a B moiety that is somehow involved in delivering the former to the target cell's cytosol, where it acts. In most instances the toxin binds through its B component to a cell membrane receptor and is endocytosed; ultimately, its A component is then translocated across the membrane of an intracellular vesicle into the cytosol. In some cases (e.g., ricin and cholera toxin), translocation is accomplished by co-opting the cell's own translocation machinery (see Hazes and Read, 1997), but in other cases (e.g., diphtheria toxin, i.e., DT, and botulinum toxin, i.e., BT), a portion of the B component is directly involved in the process. In fact, all of the translocation machinery for DT and BT is built into their B portion, so that the A portion can be translocated across a planar lipid bilayer without the aid of any cellular components (Oh et al., 1999; Koriazova and Montal, 2003). A part of the B portion of these self-translocating toxins forms channels in planar lipid bilayer membranes at low pH values mimicking the conditions of acidic cellular vesicles (e.g., Hoch et al., 1985), but whether the channel is a conduit for the A portion or is a ''discarded wrapper'' (Misler, 1984) is not clear. Equally unclear is the structure of these channels and the possible role of lipid in their architecture (Gordon and Finkelstein, 2001).

Anthrax toxin is also classified as an AB toxin, but differs from the others in that it has two A portions instead of one: edema factor (EF) and lethal factor (LF). Also, unlike DT and BT, in which the A and B portions are covalently linked, EF (89 kDa), LF (90 kDa), and PA (83 kDa) (the protective antigen B component) are separate molecules. As with the other AB toxins, the B portion (PA) first binds to a cell receptor. A furin-like protease then cleaves a 20-kDa fragment from the N-terminal end of PA, leaving PA_{63} attached to the membrane, which then oligomerizes and thereby generates and exposes binding sites for EF and LF. After endocytosis of PA_{63} with its bound EF and/or LF, these enzymes are translocated across the membrane of an acidic vesicle into the cytosol (for a general review of anthrax toxin see Collier and Young, 2003). In a manner analogous to the B portions of DT and BT, PA_{63} forms channels in planar bilayer membranes under mildly acidic conditions (Finkelstein, 1994). One can therefore ask 1), can EF and LF, like the enzymatic portions of DT and BT, be translocated across a planar bilayer in the absence of any cellular components, and if so, 2), does the channel act as a conduit for this process?

There are two advantages that anthrax toxin has over DT and BT in addressing this second question. First, channel formation can be separated from translocation; that is, one can first form the channel with PA_{63} and then add EF or LF. Thus, if under these circumstances translocation occurs, it is clear that the channel is not a discarded wrapper. Of course, one can also first form the channel from the B portion of DT or BT and then add their respective A portions, but there is little chance of observing translocation of A, even if it did occur, under these circumstances. This is because there is no receptor-binding site on the DT and BT channels for their respective A portions, as there is on the PA_{63} channel of anthrax toxin. Once EF or LF has bound to the PA_{63} channel (or, as in the case of DT and BT, the A component is already covalently linked to the B channel), the enzyme's concentration is orders-of-magnitude larger than can be achieved by adding free A to solution. The second advantage of anthrax

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toxin over DT and BT is that there is structural information about the PA_{63} channel. It is a heptameric, 14-stranded β-barrel with a limiting diameter of ~12 Å somewhere in its length of ~100 Å (Petosa et al., 1997; Blaustein and Finkelstein, 1990; Nassi et al., 2002), and there are good reasons to believe that its structure resembles in many ways that of the channel formed by Staphylococcal α-toxin (Song et al., 1996). The binding site for EF and LF spans the interface between two subunits, and the channel can bind up to three molecules of EF and/or LF (Cunningham et al., 2002; Mogridge et al., 2002).

In this article we report our investigations of the interactions in planar lipid bilayers of the PA_{63} channel with LF_N, the 263-residue N-terminal piece of LF that contains the region that binds to the channel. At positive voltages ($\geq$40 mV) LF_N is translocated across the membrane, and does so by going through the channel N-terminal end first.

MATERIALS AND METHODS

Protein preparation: Purification of wild-type PA from BL21(DE3) and formation of PA heptamer, $(PA_{63})_7$, were done as described earlier (Sellman et al., 2001; Cunningham et al., 2002). Trypsin-nicked PAG323C (nPAG323C) was made as described by Benson et al. (1998). LF_N was prepared as described by S. Zhang, A. Finkelstein, and R.J. Collier (unpublished). Its N-terminal H_6-tag was removed by thrombin cleavage, leaving four exterior residues, Gly-Ser-His-Met, at the N-terminus. Biotinylation of LF_N was done as described by S. Zhang, A. Finkelstein, and R.J. Collier (Unpublished). LF_N-DTA (LF_N with diphtheria toxin A chain attached to its C-terminus) was kindly provided by Dr. Steve Juris. Protein concentrations were determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA).

Bilayers were formed at room temperature by the brush technique of Mueller et al (1963) across either a 0.5-mm hole in a Teflon partition or a 0.2-mm diameter hole in a Delrin cup (Warner Instruments, Hamden, CT). Membranes separated two compartments of either 3 ml or 1 ml containing symmetric solutions of 100 mM KCl, 25 mM potassium succinate, and 1 mM EDTA, pH 5.5, which could be stirred by small magnetic stir bars. The membrane-forming solution was 3% diphytanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in *n*-decane. After the membrane formed, $(PA_{63})_7$ was added to the *cis* compartment, to a final concentration of ~1 ng/ml (~2 pM), and a steady-state current level was reached (at a transmembrane voltage of +20 mV) after several minutes. LF_N, or modified forms of it, were then added to the *cis* compartment to a final concentration of ~3 nM. The contents in this *cis* compartment could be slowly perfused to remove LF_N by the simultaneous addition and removal of solution through a coupled pair of syringes; the exchange of >6 volumes was accomplished in several minutes. Assuming complete mixing of *cis* compartment contents by the continuous stirring, this brought the LF_N concentration down to <0.25% of its original value. All experiments were done under voltage-clamp conditions; voltages are those of the *cis* solution (to which protein was added) with respect to that of the *trans* solution, which was held at virtual ground. Current responses were filtered at 10 Hz and recorded either on a Narco physiograph chart recorder (Houston, TX) or filtered at 100 Hz and recorded on a computer with Axograph 4.0 software (Foster City, CA).

The sulfhydryl-specific reagents N-(β-D-glucopyranosyl)-n′-[(2-methanethiosulfonyl)ethyl]urea (MTS-5-glucose), 2-aminoethyl methanethiosulfonate hydrochloride (MTS-EA), and [2 (trimethylammonium)ethyl] methanethiosulfonate bromide (MTS-ET) were purchased from Toronto Research Chemicals (North York, Canada).

RESULTS

LF_N's effect on PA_{63}-induced conductance

With the transmembrane voltage held at +20 mV, the addition of $(PA_{63})_7$ to the *cis* solution (final concentration 0.2–6 pM) causes the membrane conductance (I/V) to rise over several minutes until a more-or-less steady level is reached; this conductance results from the insertion of several hundreds or thousands of $(PA_{63})_7$ channels into the membrane. After this steady state is reached, the application of negative voltages leads to a decline in conductance (the larger the voltage the greater and faster the decline), and similarly large positive voltages also lead to a conductance decline. For a given absolute magnitude of voltage, negative voltages have a larger effect than positive ones (Finkelstein, 1994). The quantitative aspects of this voltage gating in terms of magnitude and timescale can vary greatly from membrane to membrane, and its mechanism is not understood. In some membranes there may be very little gating between ±70 mV, whereas in others there can be significant gating between ±20 mV. In the experiments reported here, this intrinsic voltage gating of $(PA_{63})_7$ channels was small over the voltage range examined and in any case was trivial compared to the effects produced upon addition of LF_N.

When LF_N was added to the *cis* solution (final concentration 0.3–4 nM) with the transmembrane voltage held at +20 mV, there was a precipitous 20- to 50-fold decline of conductance over several seconds (Fig. 1 *A*). If the voltage was now stepped to −20 mV there was a rapid increase of conductance (although not back to the original value before LF_N addition), and if the voltage was then returned to +20 mV, this increased conductance rapidly fell back to its previous level (Fig. 1 *A*). (See also S. Zhang, A. Finkelstein, and R.J. Collier, Unpublished.) Larger positive voltages gave faster declines of conductance to even lower values, sometimes followed by a subsequent rise in conductance (Fig. 1 *B*). We attribute the declines in conductance to the entry of LF_N into the $(PA_{63})_7$ channels with resultant channel block, and the increases in conductance to the exit of LF_N from the channels, which unblocks them. It is not possible, however, to obtain any quantitative insight into these processes from such experiments, as they are confounded by the continual presence of LF_N in the *cis* solution; this LF_N can reblock channels from which LF_N has exited, and thereby attenuate or completely eliminate the conductance rise associated with LF_N exit from the channels.

Consequently, after the effect of LF_N on conductance had reached a steady state, we perfused LF_N (and incidentally PA_{63}) out of the *cis* compartment, while holding the voltage at +20 mV. (The washout of $(PA_{63})_7$ channels from the membrane was very slow, with a half-time of over an hour, so that the number of channels in the membrane remained essentially constant during a single perfusion experiment.) At the end of the perfusion, the conductance was slightly higher than at the beginning and continued to rise slowly

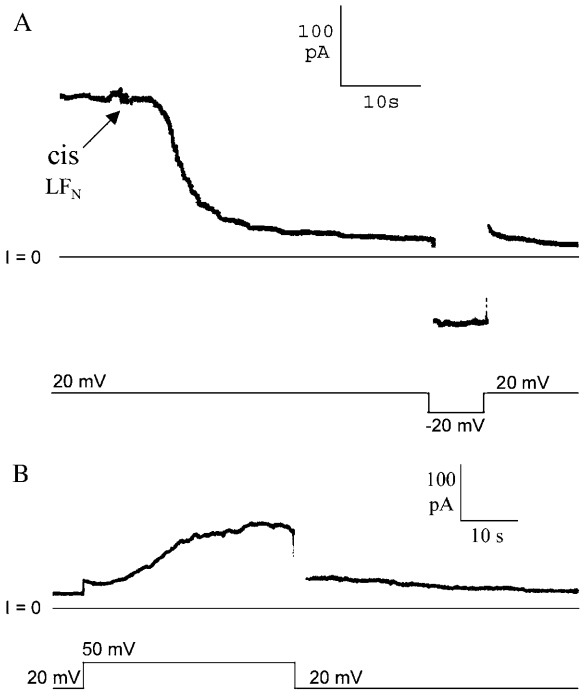

FIGURE 1 The effect of LF_N on PA_{63}-induced conductance. (*A*) Before the start of the record, $(PA_{63})_7$ was added to the *cis* compartment (final concentration 0.2 pM). After several minutes the conductance reached a steady state. With the voltage held at +20 mV, LF_N was added to the *cis* compartment to a concentration of 0.33 nM, causing the conductance to precipitously decline. When the voltage was stepped to −20 mV, the current "instantaneously" (within 0.1 s) increased, although not to the original value, and when the voltage was returned to +20 mV, the current rapidly declined. (The level reached is lower than that before the −20 mV stimulus, because the full effect of LF_N had not yet been achieved.) (*B*) When the voltage was stepped from +20 mV to +50 mV (after addition, in a different experiment, of LF_N to a concentration of 0.33 nM), the ''instantaneous'' current jump was subOhmic and continued to decline, before rising to a higher level. When the voltage was returned to +20 mV, the current (after an Ohmic drop) declined to its previous level.

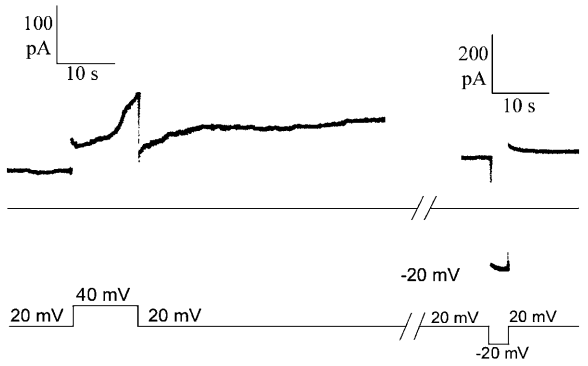

FIGURE 2 The response of $(PA_{63})_7$ channels treated with LF_N, after perfusion of LF_N (and PA_{63}) from the *cis* compartment. Before the start of the record, $(PA_{63})_7$ was added to the *cis* compartment (final concentration 0.4 pM), and after a steady-state conductance was reached, LF_N was added to the *cis* compartment (with the voltage held at +20 mV) to a final concentration of 3.3 nM. The conductance rapidly declined as in Fig. 1 *A* and eventually reached a value of only 2% of that before LF_N addition. With the voltage held at +20 mV, the *cis* compartment was slowly perfused to remove the LF_N (and incidentally the PA_{63}); during this time the conductance slowly increased, reaching a value of ~6% of that before LF_N addition, at which point the record begins. When the voltage was stepped from +20 to +40 mV the conductance is seen to rise, and when the voltage was stepped back to +20 mV it continued to rise for several seconds before leveling off. When the voltage was stepped from +20 to −20 mV, the conductance rapidly increased, but when it was returned to +20 mV, the conductance did not continue to increase nor remain at this higher level, but instead declined to a level only slightly larger than before. (The break in the record is 40 s.)

over time, indicating that LF_N slowly dissociates from the channels at +20 mV. When the voltage was stepped to −20 mV there was a very rapid increase of conductance, but when the voltage was returned to +20 mV, the conductance declined from this value to a value somewhat higher than before the switch to −20 mV (Fig. 2). Thus, most of the channels that became unblocked at −20 mV still had LF_N attached to them, which could reblock them when the voltage was returned to +20 mV. In contrast, when the voltage was stepped to +40 mV, there was a slow S-shaped increase in conductance, and when the voltage was returned to +20 mV, the increase continued for a few seconds before leveling off (Fig. 2). Thus the channels that became unblocked at +40 mV no longer had LF_N attached to them, and some that were still blocked at +40 mV became unblocked at +20 mV and lost the LF_N attached to them.

How do we interpret these results? As illustrated in Fig. 3, we believe that LF_N binds to its cognate sites on the $(PA_{63})_7$ channel and can be driven into the channel lumen at +20 mV to block it. At −20 mV, LF_N exits the channel lumen to the *cis* side, thereby unblocking it, but remains attached to the channel, from which it slowly dissociates into the *cis* solution; it therefore can reblock the channel when the voltage is returned to +20 mV. At larger positive voltages (≥+40 mV), LF_N is driven all the way through the channel and out into the *trans* solution, thus unblocking it. When the voltage in this case is returned to +20 mV, there is no LF_N still attached to the channel to reblock it. The rise in current for a few seconds upon the return of the voltage to +20 mV represents channels that become unblocked as LF_N continues to pass through them to the *trans* solution.

The kinetics and voltage dependence of LF_N translocation at positive voltages are interesting (Fig. 4). When the voltage was stepped from +20 mV to larger values (≥+40 mV), the conductance rise was S-shaped, and its rate increased relatively steeply with voltage. For example, the half time of unblocking was ~36 s at +40 mV and 11 s at +50 mV.

LF_N translocation across $(PA_{63})_7$-treated membranes occurs in an N-terminal to C-terminal direction

S. Zhang, A. Finkelstein, and R.J. Collier (Unpublished) showed that LF_N's N-terminal end entered and blocked the

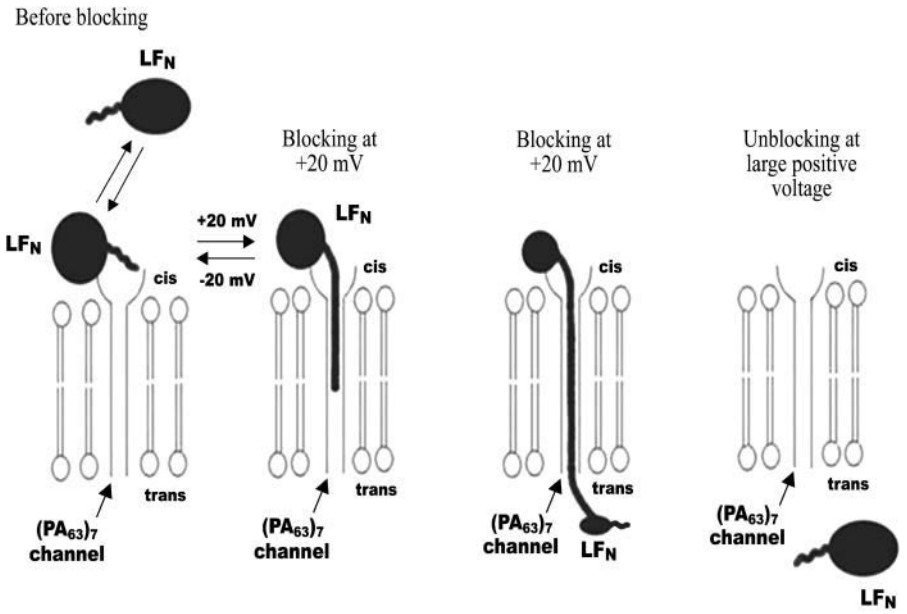

FIGURE 3 Cartoon of the interaction of LF_N with the $(PA_{63})_7$ channel and the effect of voltage on this interaction. LF_N is diagramed as a folded structure with the N-terminal 26 residues disordered (Pannifer et al., 2001) and thus able to enter the channel and block it at +20 mV. A voltage of −20 mV drives the N-terminal end out of the channel and thereby unblocks it, but LF_N can still remain attached to the channel and only slowly exchanges with free LF_N in the *cis* solution. Under the applied +20 mV, LF_N can further unfold and traverse the entire channel, with part of its N-terminal end re-emerging on the *trans* side and refolding in the *trans* solution. This also occurs at larger positive voltages (not shown). At these larger voltages, this process ultimately leads to complete translocation of LF_N into the *trans* solution.

$(PA_{63})_7$ channel at +20 mV and could even cross the membrane to the *trans* solution. We proposed that this initiates translocation of LF_N in an N-terminal to C-terminal direction, and in the preceding section we saw that LF_N translocation across the membrane does indeed occur at larger positive voltages. In this section we present experiments demonstrating that translocation is prevented if the C-terminal end is trapped on the *cis* side, thus confirming our proposed directionality of translocation.

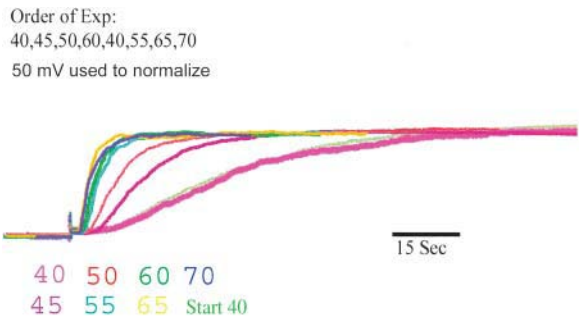

FIGURE 4 Kinetics of LF_N translocation. These are normalized conductance versus time records of successive additions of LF_N (final concentration of 3.3 nM) to the *cis* compartment, followed by perfusion of that compartment to remove LF_N, followed then by a step of voltage from +20 mV to the indicated voltage. The experiment was begun by adding $(PA_{63})_7$ to the *cis* compartment (final concentration 1.4 pM) and then waiting until a steady-state conductance level was reached at +20 mV before the first addition of LF_N. (Note that after the first perfusion, there was no free $(PA_{63})_7$ left in the *cis* compartment during the taking of these records.) The order of the voltage steps was 40, 45, 50, 60, 40, 55, 65, and 70 mV. The agreement of the two +40 mV records illustrates the reproducibility of the kinetics. At the end of the experiment, 2 h after the start, the conductance was 25% of that before the first addition of LF_N—a reflection of the slow washout of the $(PA_{63})_7$ channels from the membrane. Note the S-shaped kinetics and their voltage dependence.

Biotin attached to the C-terminal end of LF_N (LF_N-biotin)

With streptavidin prebound to LF_N-biotin, channels were still blocked at +20 mV (S. Zhang, A. Finkelstein, and R.J. Collier, Unpublished), indicating that LF_N does not enter the channel C-terminal end first. Furthermore, these channels were not unblocked at larger positive voltages (Fig. 5), consistent with streptavidin anchoring the C-terminal end of LF_N to the *cis* side of the membrane. This interpretation was confirmed by the addition of tris-carboxyethylphosphine to

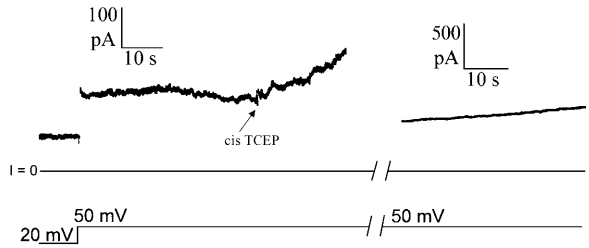

FIGURE 5 Evidence that LF_N enters the $(PA_{63})_7$ channel N-terminal end first. Before the start of the record, $(PA_{63})_7$ was added to the *cis* compartment (final concentration 6 pM). After a steady state was reached, LF_N with biotin at its C-terminal end (LF_N-biotin) was added to the *cis* compartment to a final concentration of 3.3 nM (causing a large decline in conductance); subsequently streptavidin was added to the *cis* compartment (final concentration 10 μg/ml), and the *cis* compartment was then perfused with the voltage held at +20 mV. The record begins shortly after the end of the perfusion. Note that when the voltage was stepped from +20 to +50 mV, there was no increase of conductance, indicating that the C-terminal end of LF_N (bound with streptavidin) is still on the *cis* side. This was confirmed by the addition of tris-carboxyethylphosphine (final concentration 4.5 mM) to the *cis* compartment. This reduced the disulfide bond linking biotin (with its attached streptavidin) to the C-terminal end of LF_N and thereby allowed LF_N to be translocated through the channel to the *trans* solution; the consequent unblocking of the channels is reflected in the continuous rise of conductance. (The break in the record is 60 s.)

the *cis* solution, which reduced the disulfide bond linking biotin (with its attached streptavidin) to LF_N, and hence now allowed LF_N to be translocated to the *trans* side (Fig. 5).

Diphtheria toxin A chain (DTA) attached to the C-terminus of LF_N (LF_N-DTA)

Adenine binds to DTA at its active site, stabilizing its native conformation (Kandel et al., 1974), so that adenine in the *cis* solution should anchor the C-terminal end of LF_N-DTA to the *cis* side of the membrane and thereby inhibit unblocking of LF_N-DTA at large positive voltages. This is precisely what we saw (Fig. 6). As expected, when adenine was removed from the *cis* solution, LF_N-DTA was now translocated to the *trans* solution and unblocking occurred (Fig. 6). LF_N-DTA was as active as LF_N in blocking $(PA_{63})_7$ channels, but the unblocking rate was slower (compare the unblocking rate at +60 mV in Fig. 6 with that in Fig. 4).

LF_N is translocated through the $(PA_{63})_7$ channel

The results presented in the preceding sections show that LF_N is translocated across PA_{63}-treated membranes. Although the most obvious pathway for this process is through the $(PA_{63})_7$ channel, it is conceivable that a path outside of the channel lumen has been created and what we have called *blocking* and *unblocking* of the channel is actually gating that occurs when LF_N interacts with the channel. The following experiment demonstrates that the pathway for LF_N translocation is through the channel lumen.

Translocation of LF_N through the channel should be slowed or prevented by any manipulation that narrows the channel lumen. The former indeed happened when we reacted the cysteines lining the lumen of channels formed by PA_{63}G323C (Benson et al., 1998) with MTS-5-glucose; the unblocking rate at +50 mV was dramatically slowed after the reaction (Fig. 7). A similar slowing of unblocking rate was seen after reaction with MTS-EA and MTS-ET (data not shown).

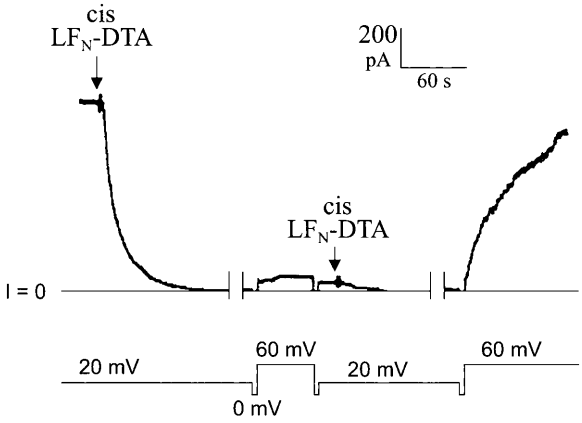

FIGURE 6 Translocation of LF_N-DTA (LF_N with diphtheria toxin A chain attached to its C-terminus). Before the start of the record, $(PA_{63})_7$ was added to the *cis* compartment (final concentration ~4 pM). The record begins, after a steady state was reached, when LF_N was added to the *cis* compartment (final concentration 4 nM), causing a large fall in conductance. During the first break in the record, the *cis* compartment was perfused with the bathing solution supplemented with 1 mM adenine. When the voltage was stepped to +60 mV, there was virtually no rise in conductance (i.e., no unblocking of the channels). A second addition of LF_N-DTA (final concentration 4 nM) was added to the *cis* solutions to further block the channels. During the second break in the record, the *cis* compartment was again perfused with the bathing solution, except this time it did not contain adenine. When the voltage was now stepped to +60 mV, the conductance rose with time (i.e., the channels became unblocked). Thus adenine, that stabilizes the native (folded) confirmation of DTA, inhibited the translocation of LF_N-DTA.

DISCUSSION

As we pointed out in the Introduction, the B component of several AB toxins forms channels in cell membranes and lipid bilayers, but the role of these channels in the translocation of the A component is unclear. This is particularly the case because the A component is covalently attached to the B and hence, at the time resolution of the experiments,

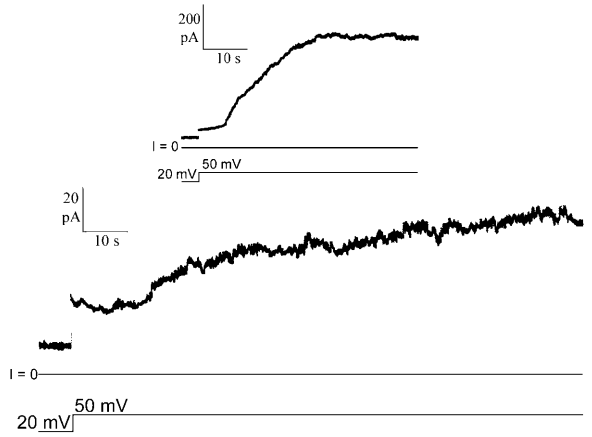

FIGURE 7 Demonstration that LF_N translocation is through the channel. (*Top*) The control. Before the start of the record nPAG323C was added to the *cis* compartment (final concentration ~200 pM). After a steady state was reached, LF_N was added to the *cis* compartment (final concentration 1.3 nM) causing a large fall in conductance, and the *cis* compartment was perfused with the voltage held at +20 mV. The record begins shortly after the end of the perfusion. When the voltage was stepped to +50 mV, the conductance rose with S-shaped kinetics, with a half-time of ~12 s; this record is in excellent agreement with previous results (e.g., Fig. 4). (*Bottom*) Before the start of the record, a subsequent addition of more nPAG323C (final concentration ~400 pM) brought the steady-state conductance up to a higher value. Addition of MTS-5-glucose to the *trans* compartment (final concentration 555 μM) with the voltage held at +20 mV caused the conductance to fall to a new steady state (reached after ~2 min) that was fivefold lower. Subsequent addition to the *cis* compartment of LF_N (final concentration 1.3 nM) again caused a large fall in conductance. The *cis* compartment was then perfused with the voltage held at +20 mV, and the record begins shortly after the end of the perfusion. Note the much slower rise of conductance here, compared to that without the MTS-5-glucose reaction (*top*), when the voltage was stepped to +50 mV.

channel formation by B and translocation of A occur concomitantly. With anthrax toxin, however, it is possible to first form the channel from the B component (PA, or more accurately, PA_{63}), and then see if translocation of the A component (LF or EF) occurs upon its subsequent addition. We have done this in planar lipid bilayers with LF_N (the 263-residue N-terminal part of LF that contains the region that binds to the $(PA_{63})_7$ channel) and demonstrated that it can be translocated across the bilayer through the $(PA_{63})_7$ channel under applied positive voltages. Thus, all of the translocation machinery is contained in this channel; no additional components, such as cellular proteins, are required for translocation.

We found that when LF_N was added to the *cis* solution in the presence of open $(PA_{63})_7$ channels, with the transmembrane voltage held at +20 mV (with reference to the *trans* solution), there was a large decrease in the PA_{63}-induced conductance, which we interpreted as a blocking of the $(PA_{63})_7$ channels (Fig. 1; see also S. Zhang, A. Finkelstein, and R.J. Collier, Unpublished). When LF_N was perfused out of the *cis* compartment (with the voltage held at +20 mV) and the voltage was then stepped to −20 mV, there was a rapid unblocking of the channels, but when the voltage was returned to +20 mV, most of the channels became reblocked (Fig. 2). We interpreted this to mean that LF_N was driven out of the channels back to the *cis* side by the −20 mV, but it remained attached to the channels; that is, the dissociation of LF_N from its binding site on the channels is slow (Fig. 3). In contrast, if the voltage was stepped to +40 mV or higher, the channels were unblocked, as at −20 mV, but remained unblocked when the voltage was returned to +20 mV (Fig. 2). (In fact, if the voltage was returned to +20 mV before all of the channels were unblocked, channels continued to unblock for several seconds—a phenomenon we comment upon later.) We interpreted this to mean that LF_N was translocated across the membrane into the *trans* solution (Fig. 3).

Consistent with, and extending this interpretation, are the following:

1. The addition of streptavidin to the *cis* solution prevented unblocking at large positive voltages of LF_N-biotin (LF_N with biotin attached at its C-terminal end; see Fig. 5).
2. The addition of adenine, which stabilizes the native structure of diphtheria toxin's A chain, to the *cis* solution prevented unblocking at large positive voltages of LF_N-DTA (LF_N with diphtheria toxin's A chain attached to its C-terminus; see Fig. 6).
3. Biotin-LF_N (LF_N with biotin attached at its N-terminus) pre-incubated with streptavidin did not block the channels, whereas LF_N-biotin preincubated with streptavidin was perfectly capable of blocking channels (S. Zhang, A. Finkelstein, and R.J. Collier, Unpublished).
4. The unblocking rate at −20 mV of H_6-LF_N (LF_N with a H_6-tag attached at its N-terminus) was slowed by Ni^{2+} in the *trans* solution (S. Zhang, A. Finkelstein, and R.J. Collier, Unpublished).
5. Unblocking at −20 mV of biotin-LF_N-DTA was inhibited by streptavidin in the *trans* solution (S. Zhang, A. Finkelstein, and R.J. Collier, Unpublished).

All of these results are consistent with LF_N binding to its cognate sites on the $(PA_{63})_7$ channel and entering the channel N-terminal end first, thereby blocking the channel; negative voltages drive the LF_N out of the channel back to the *cis* side, whereas large (≥40 mV) positive voltages drive LF_N all the way through the channel into the *trans* solution (Fig. 3). Introducing bulky groups in the channel lumen slowed translocation (Fig. 7), thus confirming that the translocation pathway is through the channel lumen.

There are several aspects of LF_N translocation through $(PA_{63})_7$ channels that deserve comment.

Mechanism

The simplest translocation mechanism to explain our results is that positive voltages electrophorese LF_N through the channel. There is, however, a problem with this proposition. Although the N-terminal two-thirds or so of LF_N has a net positive charge, and therefore can be driven into the channel by positive voltages, the C-terminal one-third has a net negative charge, which would act to prevent its translocation under an applied positive voltage (Bragg and Robertson, 1989). (The disordered 26 residues at the N-terminal end of LF_N—Pannifer et al., 2001—with its net positive charge, may make it particularly suitable for the N-terminus to be driven into the channel). This problem disappears if the pK values of the aspartates and glutamates of LF_N are one or two pH units higher when LF_N is within the channel, in which case its C-terminal third would not bear a net negative charge. Other factors, however, may be involved in the translocation of LF_N, one of these being the refolding energy. Since the diameter of the $(PA_{63})_7$ channel is only ~12 Å somewhere in its length (Blaustein and Finkelstein, 1990), LF_N must unfold to traverse the lumen—certainly losing its tertiary structure and possibly also its secondary structure. Once part of the N-terminal region has been translocated to the *trans* solution, one can envision that its refolding acts as a driving force for the translocation of the rest of the molecule. This could explain why translocation and unblocking continue to proceed when the voltage is stepped back down from +40 to +20 mV (Fig. 2). We have recently found that at pH 6.6 (instead of our usual pH 5.5), channels that are blocked by LF_N are not unblocked at large positive voltages. This effect of pH on translocation could be due to either now imparting a net negative charge to the C-terminal third of LF_N or inhibiting unfolding of LF_N.

In considering the mechanism of protein translocation through the $(PA_{63})_7$ channel, we have confined ourselves in this article to LF_N translocation across a membrane separat-

ing symmetric solutions at pH 5.5. We have not dealt with the more biologically relevant molecules, whole LF or EF. Nor have we addressed translocation in the presence of a pH gradient across the membrane (e.g., pH 5.5/pH 7.2), a situation germane to that faced by LF and EF in an acidic vesicle. Finally, we have not considered the role that chaperones may play in translocating LF and EF into the cytosol. These are topics for future publications. The purpose here has been to show that the $(PA_{63})_7$ channel functions as a conduit for protein translocation that can be driven by transmembrane voltages. The magnitude of the transmembrane potential of acidic vesicles is difficult to assess, but there is no question of its sign (e.g., Van Dyke and Belcher, 1994); namely, the interior of the vesicle is positive with respect to its surroundings, which is the same polarity that drives translocation in our experiments.

Kinetics

There are two aspects of the kinetics of translocation that are noteworthy. One, is their sigmoidal nature (Fig. 4). This must mean that there are multiple (more than one) blocked states in series that have to be traversed before LF_N exits the channel. This could be a consequence of there possibly being two or three LF_Ns bound per channel in our experiments (Mogridge et al., 2002), in which case unblocking would not occur until the last one has passed through. Experiments with mutant channels that have only one LF_N-binding site, however, continue to display S-shaped kinetics (unpublished results), which means that these kinetics are intrinsic to the translocation of a single LF_N molecule. Moreover, the continued translocation that occurs for several seconds when the voltage is stepped back to +20 mV from a larger positive voltage (Fig. 2) indicates that there is more than one kinetic barrier.

A second relevant aspect of the kinetics of translocation is the timescale of several seconds. Unless one assumes an extremely slow diffusion of LF_N within the channel, translocation of this 263-residue protein should occur on a millisecond or submillisecond timescale. Indeed, translocation of RNA through the staphylococcal α-toxin channel, which bears many similarities to the $(PA_{63})_7$ channel, occurs in microseconds (Akeson et al., 1999). As we mentioned earlier, for LF_N to get through the channel lumen, the folded molecule must become unfolded under the applied voltages, and it is perhaps this step, or steps, that is rate-limiting.

The effect of sulfhydryl-specific reagents on LF_N translocation

When the cysteines in channels formed by PA_{63}G323C were reacted with MTS-5-glucose, MTS-EA, or MTS-ET, the rate of unblocking of LF_N at +50 mV was dramatically slowed (Fig. 7). Although this provides convincing evidence that the pathway for LF_N translocation is through the $(PA_{63})_7$ channel lumen, it is surprising that introduction of a relatively rigid, bulky group such as glucose did not completely block translocation. One possible reason for this is sulfhydryl exchange, which would still narrow the channel lumen by forming disulfide bonds within it, but would, in the process, expel glucose. We suspect this, because in single-channel experiments we saw up to four conductance steps (i.e., reactions) with MTS-5-glucose (unpublished results), and it is difficult to believe that the channel could accommodate four glucoses at the level of residue 323. It is more likely that each pair of reactions (two pairs giving the observed four reactions) represents the formation of R-S-S$(CH_2)_2$ glucose followed by sulfhydryl exchange, leaving R-S-S-R in the channel, where $R = PA_{63}$.

Comparison to protein translocation in other systems

As we stated in the Introduction, diphtheria toxin and botulinum toxin can also translocate their respective enzymatic domains across planar lipid bilayers (Oh et al., 1999; Koriazova and Montal, 2003), and parts of their B portion form channels (Hoch et al., 1985). But the role of these channels in the translocation process is unknown, as is their structure. More relevant to the translocation mechanism we have described for anthrax toxin is that of the autotransporters (Henderson et al., 1998). In particular, it has been shown that the translocation domain of the autotransporter NalP from *Neisseria meningitides* forms a 12-stranded β-barrel pore of diameter 10 $\times$ 12.5 Å that could act as a conduit for its passenger domain (Oomen et al., 2004); it also forms channels in planar bilayers (Oomen et al., 2004). The β-barrel of OmpF has also been proposed as the translocation pathway for colicin E3 across the *Escherichia coli* outer membrane, with BtuB acting as the receptor (Kurisu et al., 2003). Supporting this hypothesis is the finding that OmpF channels in planar bilayers are blocked by colicin E3 (Kurisu et al., 2003). In this context it is worth noting that several protein-conducting cellular channels have been incorporated into planar bilayers and shown to be blocked by proteins or peptides that are presumed to go through them (e.g., TiC 110 of the chloroplast inner envelope membrane, Heins et al., 2002; TOM 40 of the outer mitochondrial membrane, Hill et al., 1998); in one instance the voltage-dependent nature of the blockade by a 13-residue peptide suggested that the peptide was translocated through the channel (Thieffry et al., 1992).

Our present study of the translocation of LF_N through the $(PA_{63})_7$ channel is the first, to our knowledge, that presents a semiquantitative treatment of protein translocation through a channel. This system lends itself as a model for future studies of the parameters important in translocation, such as protein length, charge distribution, and the energetics of voltage-driven protein unfolding. The last mentioned is of particular interest and relevance, since the membrane potential across the inner mitochondrial membrane apparently drives protein unfolding, and moreover does so by unraveling the protein

from its positively charged N-terminal end (Huang et al., 2002).

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